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14. ABSTRACT

In this proposal, we asked if miRNA expression is altered as cells progress through the different stages of cancer. Through our microarray experiments, we have shown that many miRNAs are differentially regulated as cells progress through cancer stages. A general trend in miRNA expression emerges from this work. As cells progress toward a metastatic state, more miRNAs are down regulated, rather than up regulated. This suggests that many cellular proteins are up regulated in these cells, and this could be involved in promoting tumor growth. We propose that the mouse if a good model system for the study of breast cancer, since several miRNAs are similarly regulated in both mouse and human. Finally, we are beginning to gain insights into the mechanism of how miRNAs are involved in cell growth--studies show that some regulated miRNAs control cell death. The mis-regulation of cell death could allow cells to escape normal regulatory mechanisms for removing tumorous cells.

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INTRODUCTION

Introductory comments: This report is for a Concept grant from the DOD #BC045755. A no-cost extension was granted this past spring, so an annual report is required after the first year, with a final report due at the end of the no-cost extension.

Background: Small, noncoding RNAs (microRNAs, miRNAs) are recently discovered single-stranded RNA species of 19-23 nucleotides that are found in all animals. They function as regulatory molecules by binding to the 3'UTR sequences of target genes and inhibiting protein translation. Over 400 miRNA genes are predicted in humans and mice. From the a limited number of studies, these miRNAs have been shown to be involved in a variety of important cellular processes, such as apoptosis, viral regulation, and developmental processes, and cancer growth. Because of their mode of action, they represent a new type of gene regulation, and offer a unique opportunity to evaluate their involvement with breast cancers.

Rationale/purpose: Deletions of some human miRNA genes occur in genomic locations that show increases in many types of cancers—this suggest they may function as oncogenes or tumor suppressors. Three deleted regions containing miRNA genes show correlations with increases in breast cancer frequency. Because of their recent discovery, we know very little about their involvement in breast cancer, and we have proposed to examine their association with breast cancer. If miRNAs are linked to breast cancer, it will provide an important novel avenue for therapeutics.

Objectives: Using a series of cell lines that define the progression of breast cancer from premalignant stages to metastatic stages, we will profile the expression of known miRNA genes (a series of mouse cell lines developed by S. Rittling)(1). Since miRNA genes are non-coding, none of them are present on current microarrays—special arrays have to be built. Our hypothesis is that the expression profile of a limited set of miRNAs increases or decreases as cells progress through the defined stages of breast cancer, and thus mutations in these genes will correlate and contribute to neoplasia. Further, the target genes that are regulated by these miRNA genes may constitute a novel set of genes, thus providing new potential targets for drug therapies. When this work was begun, there are no reports of miRNA gene expression in breast cancer cells.

BODY

Task #1. ***Grow breast cancer cells and make RNA.*** For these experiments, be chose a series of mouse breast cancer cell lines (1). A high percentage of miRNA genes are conserved between mice and humans (>95%), so the mouse will provide an excellent model system for our studies. These mouse lines were derived from 129S1mice treated with a DMBA/medrxyprogesterone tumor induction protocol (see table below). These mice developed invasive mammary adenocarcinomas. A cell line derived from one of these tumors (1029) was not tumorigenic. However, 1029 cells superinfected with polyoma middle-T antigen (GP+E) are tumorigenic with a long latency. Furthermore, GP+E cells transfected with a mutant Ha-ras oncogene become highly tumorigenic upon mam-

mary fatpad injection (R3). Tumors derived from one of these ras-transfected cell lines (R3T) gave rise to metastases in liver (R3L). Thus, this panel of transformed mouse mammary cell lines represents an attractive model to study the different steps in malignant progression of mammary cancer in a syngeneic host. Finally, 4T1 cells, derived by Fred Miller, are highly metastatic *in vivo*.

Origin	Immortal	Invasive	Metastatic	Derived (Metastatic) Site
R.B. Owens	**NMuMG**			Not tumorigenic, benign cystadenomas are produced in isogenic mice
S. Rittling		**1029**		fibrosacoma, connective tissue
		GP+E	**R3**	Transfection with v-Ha-ras
			R3T	Mammary tumor
			R3L	Lung metastases
B.A. Pulaski			**4T1**	BALB/c mice

Cells were grown under conditions appropriate for each cell line using standard conditions. Cells were harvested and RNA was made from each cell line in triplicate. The Ambion mirVana™ kit was used to make small RNA fractions. The small fractions <100 nucleotides contain the miRNAs and this enrichment is necessary to get strong signals during hybridization. In the early phases of the projects, the RNA was examined on an agarose gel to examine the quality of the RNA. RNA concentrations were determined spectrophotometrically.

Task 2. Label RNA and probe microarrays. In previous work, we developed a microRNA microarray platform with our collaborators (2). This array contained all the validated miRNAs from mice, rat, and humans. Each microarray contained antisense oligonucleotide dimmers of each miRNA, which would hybridize to miRNAs in the RNA samples. Labeling of the RNA samples was done with a kit from Genisphere, Inc. In short, an adapter was added to the RNA sample, which is complementary to a sequence in embedded in a large fluorescent reporter moiety. This reporter contains hundreds of fluorescent molecules and therefore, results in high sensitivity. A reference RNA and an experimental RNA are labeled with either Cy3 or Cy5 and mixed for hybridization. In our experiments, our reference was the 1029 mouse cell line. After hybridization, samples were scanned for both red and green to determine the level of hybridization to each miRNA. Since three independent samples were collected and hybridized, we were able to use statistical approaches to determine if any two samples were significant.

Experimental issues. At the beginning of the project, we had successfully used our microarray in a series of experiments, and showed that it worked well. However, the technology was still relatively new, and we encountered some issues that slowed the work in the initial phases. The arrays were printed locally, and not by a commercial lab. During this time, some of the machinery required repair, which required some down time. Also, a few batches of chips did not pass our stringent quality controls, so they had to be reprinted. At times, if the ozone levels were too high, the quality of chips was substandard. All these problems were satisfactorily overcome, but we did not anticipate them in the

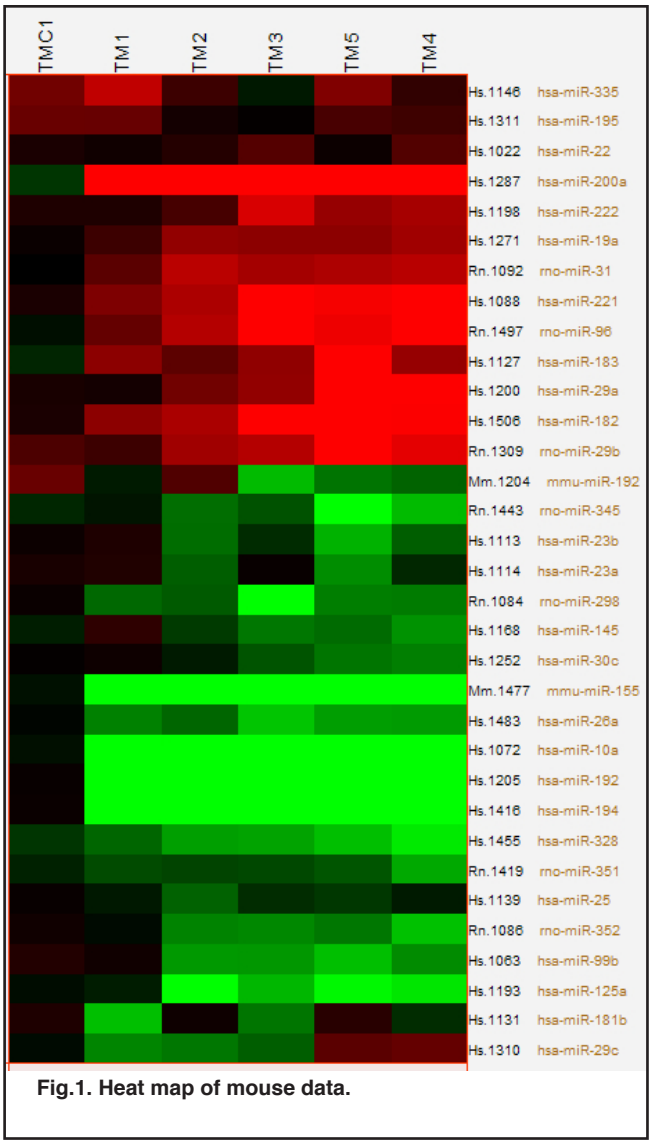

Fig.1. Heat map of mouse data.

initial phases.

To obtain a readout of each microarray, they were scanned in a microarray reader. Given that these were printed locally, all the spots had to be gridded, which proved to be time-consuming. It took some time for us to become confident in knowing how much flexibility the gridding could tolerate. There are more expensive machines that are capable of doing this, but sending samples to another university posed other complications. In the end, we developed protocols that were very reliable.

A sample of the data is shown in Fig. 1. TMC represents the control RNA from the immortal cell lines. TM1-4 are the experimental mouse cell lines (1=1029, 2=GP+E, 3=R3, 4=R3T, 5=R3L). If the miRNA expression is increased relative to the control, then the rectangles appear redder. If the expression is lower, then the rectangles appear green. Since many miRNAs are identical between human, rat, and mouse, only one oligo was synthesized for each miRNA and therefore the name of the miRNA could have either a human (hsa), mouse (mmu), or rat (rno) prefix. Notice in Fig. 1 that miR-162 is down-regulated in the more severe breast cancer lines, while miR-20C is up regulated.

Task 3. Catalog miRNA gene expression and examine genomic locations. From the results in task 2, we have identified a number of miRNAs whose expression varies from control breast tissue. This confirms our original hypothesis that miRNA expression is altered in breast cancer cells. An example is shown in Fig. 2.

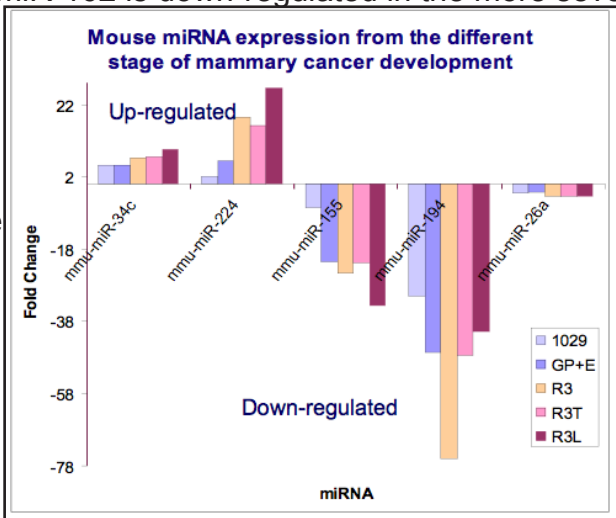

Fig.2. Examples of miRNAs whose expression changes.

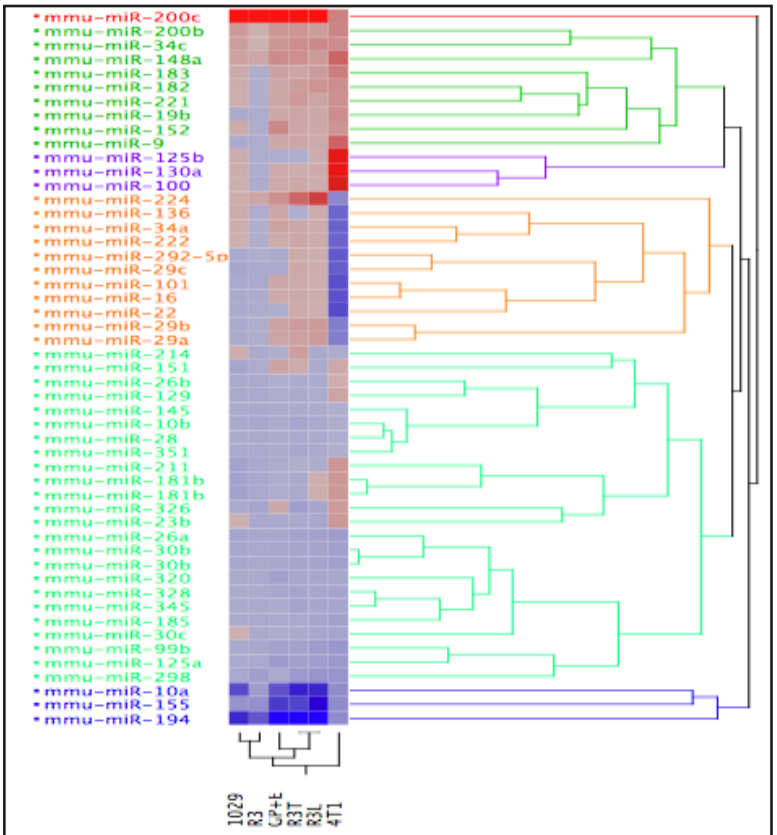

Fig.3. Examples of miRNAs whose expression changes.

These experiments allow one to determine which miRNAs change expression as a group, or as a cluster. Genes that function together may define regulatory networks and regulate a common set of regulated genes. Using clustering software, we have grouped the significantly regulated miRNAs into different groups. In Fig. 3, there are six different types of expression profiles among the significantly regulated miRNAs. Some groups show high expression in all the transformed, malignant cell lines, while some increase as the phenotypes become more severe. In some of the groups, their expression decreases as the phenotypes become more severe. In cases where the expression is reduced, we hypothesize that the genes regulated by these miRNAs go up in protein levels, and it is these cellular proteins that may be the engines that promote tumor growth.

Interestingly, we find a general trend from these data---miRNA expression goes down in more severely transformed cell lines (Fig. 4)(refer to Table 1 above for details of the cell lines). Line 1029 is a transformed mouse mammary cell, and GP+E, R3, R3T, and R3L are derived from 1029. As we examine the trends of all the miRNAs in these lines, there are an increasing number of miRNAs that are downregulated. The same is true for line 4T1, which is independently derived and has a severe cancer phenotype. This argues strongly that miRNA regulation correlates with the neoplastic state and may explain, in part, how cancerous cells avoid normal controls.

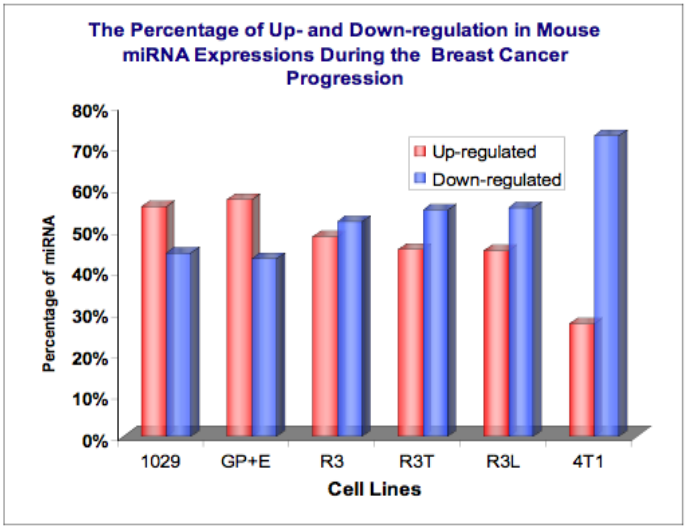

Fig.4. Global trends in expression patterns.

Next we wondered if our results were relevant to human miRNA expression in breast cancer cells. We compared our mouse results to results obtained from a series of human breast cancer cells lines that represent increasing stages of breast cancer. If there are common regulatory mechanisms operating in breast cells, then we would expect some of the miRNAs to be similarly regulated. If there are no common regulatory mechanisms present in breast cells, then we expect to find a totally different set of regulated miRNAs. Since the etiology of the human breast cancers is unknown and likely to be different from the etiology of the mouse breast cancers, any common regulated miRNAs

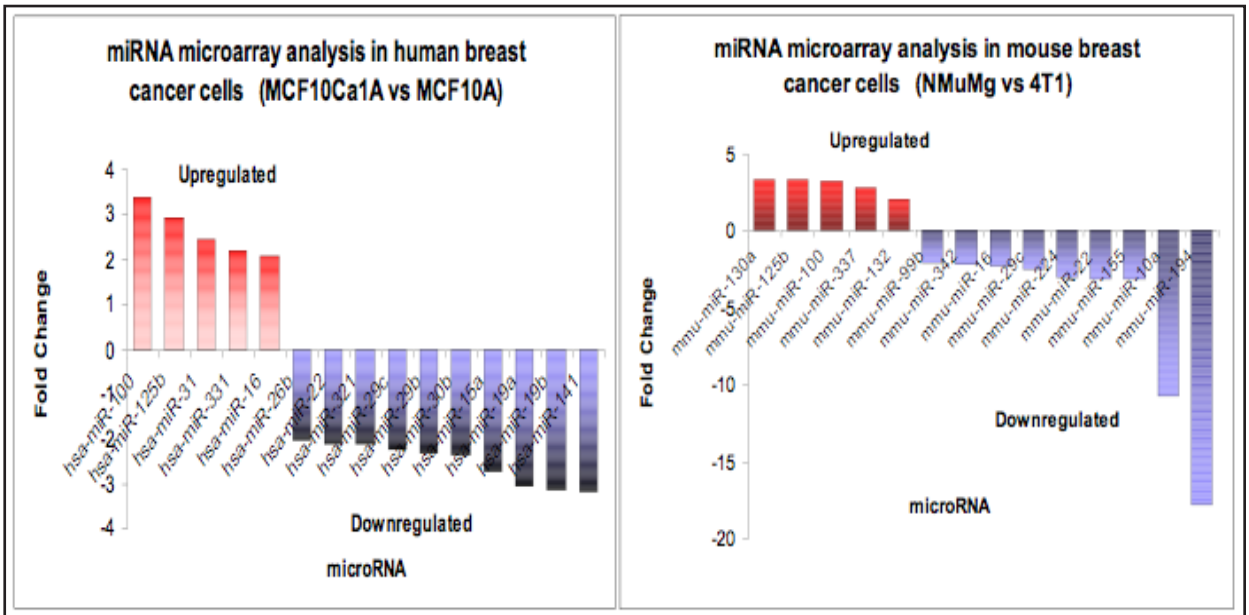

Fig.5. A comparison of mouse and human miRNA regulation.

will be even more significant. In Fig. 5, we display some of the most highly regulated miRNAs from mouse and human. Interestingly, we find that several miRNAs are regulated in a similar manner. miR-100 and miR-125b are up regulated in our samples, while miR-16 and miR-22 are down regulated. This argues strongly that breast cancers have a common set of regulated miRNAs that have been conserved and function in breast cells-these miRNAs are good candidates to pursue in future studies.

While this work was in progress, the Croce group examined the relationship between genomic rearrangement/deletions and miRNAs in various cancers (3). They found that miR-100, miR-145, miR-10b, and miR-34 are located in regions that correlate with breast and other cancers. Interestingly, we identified miR-100 as a regulated miRNA in breast cancers. The limit of this analysis is that only large chromosomal aberations are available for this type of analysis. The genomic region around each miRNA must be molecularly examined in greater detail.

Task 4. Determine potential targets for each regulated miRNA and validate. Target prediction is still a difficult task. What is currently accepted is that targets require a core sequence match of about seven nucleotides. A match of seven nucleotides occurs often in the genome, so the question remains if all these sites are *bona fide* sites. There is

some evidence that many of these seven-mers may actually be weak targets. However, this view appears to be too simple---we do not know what influence protein complexes may play in distinguishing among the seven-mer sequences. This area of research is important and researchers will continue to define real targets. There are now lists published on web sites for potential targets and we are evaluating them for the miRNAs that we found in our studies.

Task 5. Evaluate for future diagnostics. We have focused on defining what cellular process a given miRNA may participate in as a means of predicting their targets. Some studies in Drosophila and other animals have shown that miRNAs regulate apoptosis, and we wondered if this might be a general principle. We asked if the regulation of cell death is a mechanism in which some of these regulated miRNAs may function. Using human breast cancer cell lines, we have shown that miR-18a, miR-20a, miR-21, miR-214, and miR-93 regulate cell death. All these miRNAs are highly conserved in mice, and the expect that they function in a similar manner. Among the predicted targets of these miRNAs are genes known to participate in apoptosis. An interesting model is that these miRNAs alter the regulation of genes involved in apoptosis, thereby allowing cancerous cells to escape normal cellular controls. These miRNAs and their targets are good candidates for diagnostics. Efficient inhibitors exist for miRNAs and might be useful in cases where lower levels of a miRNA was desirable. miRNA pre-cursors are available, which can be used to increase levels of a given miRNA, if increased levels might lead to tumor cell death.

KEY RESEARCH ACCOMPLISHMENTS

1. showed that miRNA regulation changes in breast cancer cells vs. normal cells
2. identified a small group of miRNAs are highly regulated in breast cancer cells
3. showed that regulated miRNAs form six clusters of expression profiles
4. showed that miRNAs tend to be down regulated as cells become more cancerous
5. showed there are strong similarities between mouse and human miRNA profiles in breast cancer cells

REPORTABLE OUTCOMES

1. Yi Sun was paid in part by this grant and recently obtained a job at Bioreference, Inc. in Elmwood Park, NJ as a staff scientist. Her job requires implementation of new technologies and her experience with this project, which dealt with new technologies, was instrumental in her being offered the job.

2. Maocheng Yang, was also part of this breast cancer project. He has taken a position as a researcher at NIH in a lab where he will be involved with microarray analysis. His work on this project is directly related to the skills he needs at his new position.

3. Poster presentation to Annual Retreat of the New Jersey Cancer Institute, 2005. “Role of microRNA Genes During Breast Cancer Progression”.

4. Poster presentation to Annual Retreat of the New Jersey Cancer Institute, 2006. “microRNA Genes During Breast Cancer Progression”.

4. Talk presented to the Developmental Biology groups at Rutgers University, Oct., 2005.

5. Talk presented to the Executive Council, Cancer Institute of New Jersey, Dec., 2005

6. We have begun drafting a research manuscript of this work, and hope to submit it shortly.

7. I am planning to submit a proposal with collaborators to NIH to expand these projects.

CONCLUSIONS

Through our microarray experiments, we have shown that many miRNAs are differentially regulated as cells progress through cancer stages. These regulated miRNAs show at least six patterns of expression changes, and may represent a regulatory network. A general trend in miRNA expression emerges from this work. As cells progress toward a metastatic state, more miRNAs are down regulated, rather than up regulated. This suggests that many cellular proteins are up regulated in these cells, and this could be involved in promoting tumor growth. We propose that the mouse if a good model system for the study of breast cancer, since several miRNAs are similarly regulated in both mouse and human. Finally, we are beginning to gain insights into the mechanism of how miRNAs are involved in cell growth--studies show that some of the regulated miRNAs regulate cell death. The mis-regulation of cell death could allow cells to escape normal regulatory mechanisms for removing tumorous cells.

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APPENDICES

none.